

RAPID COMMUNICATION

## PlexinA1 expression in gastric carcinoma and its relationship with tumor angiogenesis and proliferation

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### Abstract

**AIM:** To explore the expression of PlexinA1 in gastric carcinoma and its relationship with tumor angiogenesis and proliferation.

**METHODS:** PlexinA1 mRNA and protein expressions of Semaphorin6D were measured using semi-quantity reverse transcription PCR and Western blotting in 20 cases of gastric carcinoma and corresponding normal gastric mucosa. PlexinA1, Ki-67 expression and microvessel density (MVD) were detected by immunohistochemistry in 50 cases of gastric carcinoma and 20 cases of normal gastric mucosa.

**RESULTS:** The mRNA and protein expressions of PlexinA1 in gastric carcinoma were significantly higher than that in normal gastric mucosa ( $0.71 \pm 0.37$  vs  $0.60 \pm 0.25$ ,  $P = 0.0299 < 0.05$ , and  $0.47 \pm 0.16$  vs  $0.21 \pm 0.08$ ,  $P = 0.0000 < 0.01$ ), and MVD within tumor tissues increased significantly with PlexinA1 mRNA expression ( $r = 0.8736$ ,  $P < 0.01$ ) and PlexinA1 protein expression ( $r = 0.7286$ ,  $P < 0.01$ ), and MVD of the PlexinA1 positive staining group ( $25.25 \pm 3.93$ ) was significantly higher than that of the negative group ( $19.56 \pm 1.75$ ), ( $P < 0.01$ ). Proliferation index of tumor cells within tumor tissues were positively correlated with PlexinA1 mRNA expression ( $r = 0.5420$ ,  $P = 0.014 < 0.01$ ) and PlexinA1 protein expression ( $r = 0.5024$ ,  $P = 0.024 < 0.05$ ). The proliferation index of the PlexinA1 positive staining group ( $567.69 \pm 125.61$ ) was significantly higher than that of the negative group ( $369.58 \pm 116.88$ ), ( $P < 0.01$ ).

**CONCLUSION:** PlexinA1 may play an important role in the occurrence and development of gastric carcinoma, and be related to tumor angiogenesis and proliferation.

### INTRODUCTION

Primary gastric carcinoma is one of the most common malignant tumors in China. Invasion and metastasis are the main causes for the death of cancer patients. On the other hand, invasion and metastasis of malignant tumors are closely related to the proliferation of tumor cells and angiogenesis of tumor tissues. PlexinA1 is a large transmembrane protein that is a major receptor for multiple classes of Semaphorins, either alone or in combination with neuropilins. Although PlexinA1 has pleiotropic function in formation of nervous systems, embryogenesis, angiogenesis and immunoreaction<sup>[1-4]</sup>, the function of Plexin A1 in carcinogenesis remain unrevealed. Therefore, we detected the protein and mRNA expression of PlexinA1 in gastric carcinoma and normal gastric mucosa by the semi-quantitative RT-PCR, Western blotting and immunohistochemistry in order to explore the expression of PlexinA1 in gastric carcinoma and its relationship with tumor angiogenesis and proliferation.

### MATERIALS AND METHODS

#### Patients

Twenty fresh gastric carcinoma specimens and corresponding normal gastric mucosa specimens were analyzed by RT-PCR and Western blotting. All specimens were obtained from patients (8 women and 12 men; mean age 54 years) who underwent surgery for gastric carcinoma between April 2006 and August 2006 in the General Hospital of PLA. Of the 20 patients, 9 showed high-moderate differentiation, 11 poor differentiation and 10 lymph node metastasis. Fifty paraffin-embedded gastric carcinoma specimens and twenty normal gastric mucosa specimens were collected respectively for immunohistochemistry. The patients with gastric carcinoma consisted of 21 women and 29 men with a mean age of 56 years. Of the 50 gastric carcinoma specimens, 23 were highly-moderately differentiated and

27 poorly differentiated, and 28 had lymph node metastasis. The diagnosis was confirmed by pathological examination.

### Reverse transcription PCR (RT-PCR)

Total mRNA was isolated by Trizol reagent according to the procedure of the supplier (BioDev-tech, Beijing, China), and the concentration was determined by measuring the absorbance at 260 nm and using the following data<sup>22</sup>: 1 optical density unit = 40 mg of RNA/mL. A 1.5 µg aliquot of total RNA from each specimen was reverse-transcribed into single-strand cDNA using oligo (dT)16 primer for 2 h at 37°C, each single-strand cDNA was used for subsequent PCR amplification of PlexinA1 and β-actin with the latter used as a quantitative control. The PCR was carried out in a reaction volume of 50 µL for 5 min at 95°C for initial denaturing, followed by 37 cycles of 94°C for 50 s, 55°C for 50 s, and 72°C for 1 min, then extend at 72°C for 10 min on the Authorized Thermal Cycler for PCR. The primer sequences used for amplification were 5'-TGTGGACGACCCCAAATTCTA-3' and 5'-CTGGGCAAACACGGTGAAC-3' for PlexinA1, 5'-ACACCTACCAGGGAACGGAG-3' and 3'-GCCTCTGCACATACCTGCT-5' for β-actin. The primer sequences were synthesized by Beijing Genomics Institute (China). PCR products were resolved in 2% agarose gels and visualized by staining with ethidium bromide. To quantify PCR products, the bands representing amplified products were analyzed by Quantity One Analysis Software (BIO-RAD Co. America).

### Western blotting

Expression of the PlexinA1 protein was detected using the Western blot method. After washing in ice-cold PBS, the samples were finely minced and suspended in ice-cold homogenization buffer (2 mL/g tissue), which contained protease inhibitors to minimize protein degradation. The suspension was firstly homogenized, then centrifuged at 12000 × g for 30 min at 4°C to remove the nuclei and cell debris. The supernatant (total protein extract) was collected. Equal amount (50 µg) of proteins was run on a 10% SDS-PAGE gel and electrotransferred onto Hybond-polyvinylidene difluoride membranes (Amersham, Arlington Heights, USA). The membranes were blocked for 2 h at room temperature, followed by incubation with the primary anti-PlexinA1 antibody 1:50 (Santa Cruz Co, USA) at 4°C overnight. The primary antibody was diluted in TBST containing fat-free milk. After three 10-min washes in TBST, the membrane was incubated in peroxidase-conjugated secondary antibody (Sigma, St. Louis, USA) diluted 1:800 at room temperature for 1 h. Immunoreactive proteins were visualized by autoradiogram using ECL Western blotting detection reagents (Amersham Pharmacia, Uppsala, Sweden) and exposing to X-Omat BT film (Kodak, New York, USA). Bands were analyzed by Quantity One Analysis Software.

### Immunohistochemistry

PlexinA1 multiclonal antibody 1:50 was purchased from American Santa Cruz Co. Monoclonal antibody of factor VIII and monoclonal antibody of Ki-67 (ready to use) were supplied by the Chinese Boster Co. All operations were performed according to the instructions of the

manufacturers. Positive specimens were used as positive controls and PBS in substitution of the first antibody was used as a negative control at the same time.

### PlexinA1 expression

The cytoplasm and membrane of positive cells were stained brown by PlexinA1. The result of immunostaining was recorded as negative or positive based on the expression of protein detected. At least 10 surface areas were scored, and the percentage of positive cells was calculated for each specimen. Specimens were classified as positive if more than 30% of the cells were stained at × 400 magnification.

### Proliferation index of tumor cells

The nuclei of positive cells were stained deep brown by Ki-67. The number of positive cells among 1000 tumor cells was counted per slide and taken as the tumor cell proliferation index.

### MVD of gastric carcinoma tissues

Gastric carcinoma vascular endothelial cells were stained brown. The isolated brown and yellow blood vessel endothelial cells or cell clusters in gastric carcinoma tissues were regarded as a single microvessel (Figure 1A). Areas with the highest microvessel densities were selected under × 100 microscopic magnification. Then the number of microvessels stained by factor VIII antibody in 5 vision fields was counted under × 400 magnification, respectively. The average values were taken as MVD<sup>[5]</sup>. Indistinguishable or indistinct cells were excluded.

### Statistical analysis

All data were analyzed by SPSS12.0 statistical software. *t* test, analysis of Chi-square test, and linear correlation analysis were used. *P* < 0.05 was taken as significant.

## RESULTS

### Expression of PlexinA1 mRNA by RT-PCR

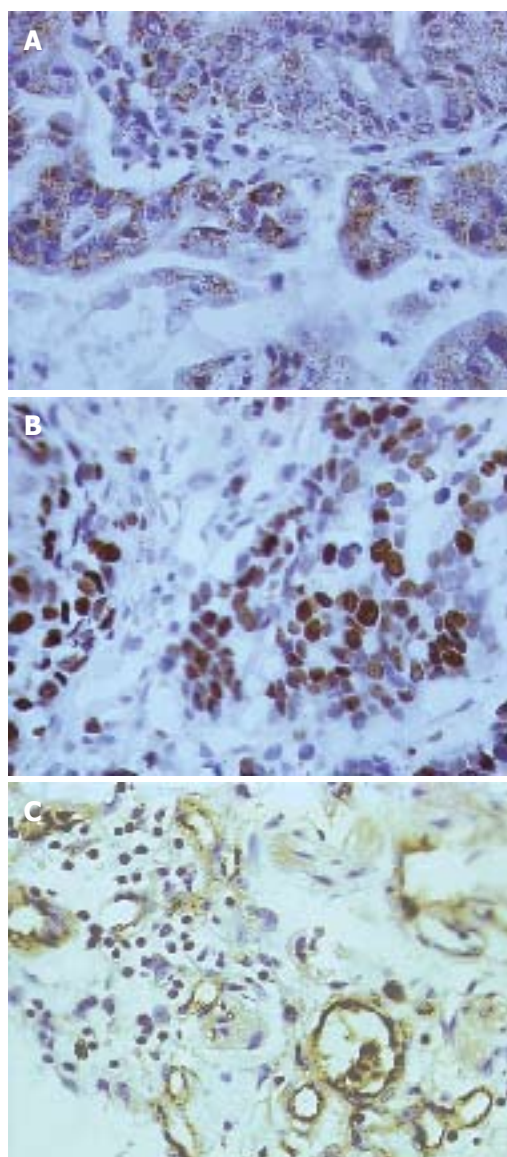
Two percent of Agarose gel electrophoresis showed a 172 bp PlexinA1 fragment by RT-PCR amplification from gastric cancer specimens and normal gastric mucosa (Figure 2). The PlexinA1 mRNA amplification was successful in all tissues. The expression level was higher in tumor ( $0.71 \pm 0.37$ ) than in normal gastric mucosa ( $0.60 \pm 0.25$ , *P* = 0.0299 < 0.05).

### Expression of PlexinA1 protein by western blotting

The affinity-purified anti-plexinA1 antibody detected a major band at 90 kD in protein extracts from all samples tested (Figure 3). The expression level was much higher in tumor ( $0.47 \pm 0.16$ ) than that in normal gastric mucosa ( $0.21 \pm 0.08$ , *P* < 0.01). This result is matched with that of RT-PCR.

### Expression of PlexinA1 by immunohistochemistry

In immunohistochemical staining, PlexinA1 located at the membrane and cytoplasm of gastric carcinoma cells appeared as brown particles. The positive expression rate of PlexinA1 in gastric carcinoma was 52%, significantly



**Figure 1** Expression of PlexinA1, Ki-67 and VIII factor in gastric carcinoma tissue (S-P × 400). **A:** PlexinA1; **B:** Ki-67; **C:** VIII factor.

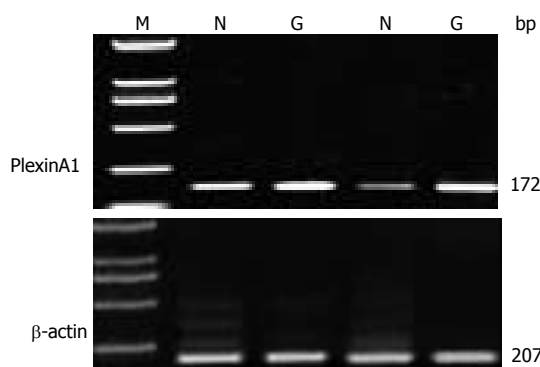
higher than that in normal gastric mucosa (25%), ( $P < 0.05$ ). However, the PlexinA1 expression level had no correlation with the age of patients, tumor size, invasion depth, differentiation degree and lymph node metastasis (Table 1).

#### Correlation between PlexinA1 and proliferation index of tumor cells in gastric carcinoma

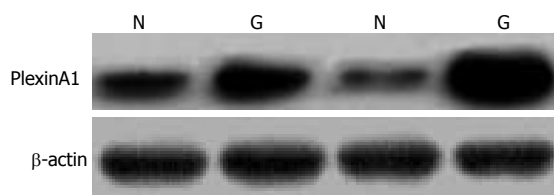
PlexinA1 mRNA and protein expression ratio of gastric carcinoma and the corresponding normal gastric mucosa (T/N) was significantly correlated with proliferation index of tumor cells in gastric carcinoma. Proliferation index of tumor cells within tumorous tissues increased significantly with PlexinA1 mRNA expression ( $r = 0.5420$ ,  $P = 0.014 < 0.05$ ) and PlexinA1 protein expression ( $r = 0.5024$ ,  $P = 0.024 < 0.05$ ). The proliferation index of the PlexinA1 positive staining group ( $567.69 \pm 125.61$ ) was significantly higher than that of the negative group ( $369.58 \pm 116.88$ ), ( $P < 0.01$ ).

#### PlexinA1 expression and the MVD of tumor tissues

PlexinA1 mRNA and protein expression ratio of gastric carcinoma and the corresponding normal gastric mucosa



**Figure 2** RT-PCR was performed to detect the expressions of PlexinA1 mRNA. M: Marker; N: Normal gastric mucosa; G: Gastric carcinoma.



**Figure 3** Western Blotting was performed to detect the protein expressions of PlexinA1. N: Normal gastric mucosa; G: Gastric carcinoma.

**Table 1** The relationship between PlexinA1 expression and clinicopathological characteristics in gastric carcinoma

| Characteristics               | PlexinA1 |          | Total | P value |
|-------------------------------|----------|----------|-------|---------|
|                               | Negative | Positive |       |         |
| Age (yr)                      |          |          |       |         |
| ≥ 45                          | 12       | 14       | 26    | > 0.05  |
| < 45                          | 12       | 12       | 24    |         |
| Size                          |          |          |       |         |
| ≥ 5 cm                        | 8        | 11       | 19    | > 0.05  |
| < 5 cm                        | 16       | 15       | 31    |         |
| Differentiation               |          |          |       |         |
| Well-moderate                 | 13       | 10       | 23    | > 0.05  |
| Poorly                        | 11       | 16       | 27    |         |
| Invasion depth                |          |          |       |         |
| Lamina and muscularis propria | 5        | 6        | 11    | > 0.05  |
| Visceral peritoneum           | 19       | 20       | 39    |         |
| Lymph node metastasis         |          |          |       |         |
| Negative                      | 10       | 12       | 22    | > 0.05  |
| Positive                      | 14       | 14       | 28    |         |

(T/N) was significantly correlated with the MVD of gastric carcinoma. MVD was elevated with the increase of PlexinA1 mRNA and protein expression. MVD within tumorous tissues increased significantly with PlexinA1 mRNA expression ( $r = 0.8736$ ,  $P < 0.01$ ) and PlexinA1 protein expression ( $r = 0.7286$ ,  $P < 0.01$ ). Also, with increase of PlexinA1 expression of gastric carcinoma tissues by immunohistochemistry, MVD rose as well. The MVD of the PlexinA1 positive staining group ( $25.25 \pm 3.93$ ) was significantly higher than that of the negative group ( $19.56 \pm 1.75$ ), ( $P < 0.01$ ).

## DISCUSSION

PlexinA1 is a large transmembrane protein that is receptor



of Semaphorins, either alone or in combination with neuropilin-1 or -2<sup>[1,6]</sup>. Despite the fact that PlexinA1 plays a crucial role in formation of the nervous system, increasing evidence attested to the significance of PlexinA1 in cardiogenesis. Some works reported that PlexinA1 has multiple functions in cardiogenesis as a receptor for the transmembrane Semaphorin, Sema6D, independent of neuropilins, and it plays a critical role in cardiac morphogenesis by regulating epithelial cell migration<sup>[2,7]</sup>. These findings suggest that PlexinA1 may regulate angiogenesis *in vivo*, and raise the intriguing possibility that PlexinA1 may play a role in tumor-induced angiogenesis.

As it is well known, the growth and metastasis of tumors require induction of angiogenesis<sup>[8,9]</sup>. Without the ability to induce angiogenesis, most neoplasms would fail to grow > 2 mm in diameter or metastasize, including gastric carcinoma. We detected the mRNA and protein expression of PlexinA1 in gastric carcinoma and normal gastric mucosa by the semi-quantity RT-PCR and Western blotting. The results showed that the expression of PlexinA1 in gastric carcinoma was significantly higher than those in normal gastric mucosa. We further detected the expression of PlexinA1 in gastric carcinoma and normal gastric mucosa by immunohistochemistry. The positive expression rate of PlexinA1 in gastric carcinoma was significantly higher than that in normal gastric mucosa. However, the PlexinA1 expression level was not correlated with the age of patients, tumor size, invasion depth, differentiation degree, and lymph node metastasis. These results suggest that PlexinA1 may play an important role in the occurrence and development of gastric carcinoma. MVD and Ki-67 are important index of judging tumor angiogenesis and tumor cell proliferation<sup>[10-12]</sup>.

Under the effect of angiogenesis and proliferation factors, tumor and endothelial cells proliferate and migrate to form new blood vessel networks and induce tumor invasion and metastasis. This study showed that MVD within tumor tissues increased significantly with PlexinA1 mRNA and protein expression, and PlexinA1 expression level of cancer tissues was positively correlated with Ki-67. These results suggest that PlexinA1 may contribute to tumor angiogenesis and tumor cell proliferation through binding its ligands named Semaphorins.

In conclusion, this is the first investigation about the expression of PlexinA1 in gastric carcinoma and its clinicopathological significance. The results of our study shed some light on the pathogenesis of gastric carcinoma, and may represent a new therapeutic target for gastric carcinoma treatment.

## COMMENTS

### Background

Primary gastric carcinoma is one of the most common malignant tumors in China. Invasion and metastasis are the main causes for the death of cancer patients, and invasion and metastasis of malignant tumors are closely related with proliferation of tumor cells and angiogenesis of tumor tissues. PlexinA1 is a large transmembrane protein that is a major receptor for multiple classes of Semaphorins, and has pleiotropic function in formation of nervous systems, embryogenesis, angiogenesis and immunoreaction, yet the function of PlexinA1 in carcinogenesis has not been intensively studied, including in gastric carcinoma.

### Research frontiers

Experiments have been employed to study the expression of PlexinA1 in gastric carcinoma and its relationship with tumor angiogenesis and proliferation. These studies show that the expression level in gastric carcinoma is higher than that in normal gastric mucosa, and is positively related to tumor angiogenesis and proliferation.

### Innovations and breakthroughs

This is the first investigation about the expression of PlexinA1 in gastric carcinoma and its clinicopathological significance. PlexinA1 is found positively related to tumor angiogenesis and proliferation, which shed some light on the pathogenesis of gastric carcinoma.

### Applications

This study may represent a new therapeutic target for gastric carcinoma treatment.

### Peer review

The authors studied the expression of PlexinA1 in gastric carcinoma and its relationship with tumor angiogenesis and proliferation, and showed that the expression level in gastric carcinoma is higher than that in normal gastric mucosa, and is positively related to tumor angiogenesis and proliferation, which may be useful in basic research of gastric carcinoma, and provide a new thought about gastric carcinoma treatment.

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